

Report for 2003AZ15B: Selection of High Performance Microalgae for Bioremediation of Nitrate-Contaminated Groundwater

- Dissertations:
 - Natalie Case. Screening and characterization of high-performance microalgae for bioremediation of nitrate-contaminated waters. M.S. Dissertation (in process) School of Life Sciences, Arizona State University, Tempe, Arizona.
 - Mike Bellefeuille. Microalgae-based nitrate bioremediation: from laboratory to pilot scale. B.S. Dissertation (in process). School of Life Sciences, Arizona State University, Tempe, Arizona.
- Conference Proceedings:
 - Natalie Case, Milton Sommerfeld, and Qiang Hu, 2004, A Search For High Performance Microalgae To Remediate Nitrate-Contaminated Groundwater: Concept And Preliminary Results. 48th Annual Meeting of the Arizona-Nevada Academy of Sciences, April 10, 2004, Midwestern University, Glendale, Arizona (Poster).
 - Mike Bellefeuille, Qiang Hu, and Milton Sommerfeld, 2004, Removal Of Nitrate From Agriculture Runoff Using The Green Alga *Scenedesmus* sp. 48th Annual Meeting of the Arizona-Nevada Academy of Sciences, April 10, 2004, Midwestern University, Glendale, Arizona (Poster).

Report Follows

A. Problem and Research Objectives:

Clean and safe water is a precious and vulnerable resource. In Arizona, more than 40% of drinking water comes from groundwater. Over 1,000 wells across the State exceed the maximum contaminant level of 10 mg L^{-1} for nitrogen as nitrate in drinking water set by the US EPA. Major pollutant sources in Arizona include agricultural activities, wastes from industries, leaking underground storage tanks, septic tanks, landfills, mining and wastewater treatment plants. Many of the groundwater quality problems are located in the Phoenix and Tucson metropolitan areas, but groundwater quality problems are found in all of Arizona's 10 watersheds. Particularly, large portions of aquifers within the Salt River Valley, including areas in Glendale, Mesa, Chandler and Phoenix, contain groundwater with nitrate concentrations high enough to render the water unfit for potable use. In addition, high nitrate levels occur in Marana, St. David, Quartzsite, Bullhead City, Lake Havasu City and other areas. Septic tank discharges are common nitrate sources in rural areas of Arizona and have contaminated drinking water wells. Quartzsite, Bullhead City and Lake Havasu City are just a few locations with documented nitrate problems from septic tanks (*ADEQ's FY '02 Groundwater Assessment*).

High levels of nitrate in groundwater pose a serious health risk for some of Arizona's residents. It can be fatal to infants when nitrate is reduced to nitrite, and the latter combines with hemoglobin in the blood to form methemoglobinaemia and leads to a condition known as "blue baby syndrome" (Gangolli et al. 1994). Reduction of nitrate to nitrite can also be a risk to adults deficient in glucose-phosphate dehydrogenase. Moreover, nitrite can react with secondary amines or amides in water or food to form *N*-nitroso compounds that are potential animal carcinogens (Shank 1975; Pontius 1993). Long-term consumption of drinking water containing nitrate concentrations of $\geq 18 \text{ mg L}^{-1}$ was reported to increase the risk of non-Hodgkin's lymphoma (Ward et al. 1996).

Nitrate removal from groundwater may be accomplished by microbial-based nitrification and denitrification, or chemically and physically-based technologies (such as ion exchange, reverse osmosis, electrodialysis and catalytic denitrification) (Kapoor and Viraraghavan 1997). However, these treatment processes are often difficult and expensive. They require input of external energy sources (e.g., electricity, organic carbon) and/or chemical additives, and generate concentrated waste-streams that then must be disposed. Shortage of surface water supplies coupled with a rapid increase in population places constant pressure on Arizona's cities and water supply utilities to treat and use available groundwater. Development of innovative, environmentally friendly and cost-effective sustainable technologies for treating nitrate-contaminated groundwater is becoming increasingly urgent.

Groundwater nitrate removal by engineered microalgal systems is an advanced concept. Microalgae require mostly simple mineral nutrients, such as nitrogen, phosphorous and inorganic carbon for growth and reproduction. By utilizing sunlight, microalgae convert, through photosynthesis, nitrate into organic compounds (such as

proteins). Microalgae can exhibit growth rates that are an order of magnitude higher than other plants due to their extraordinarily efficient light and nutrient utilization. By taking advantage of various designs of engineered microalgal photobioreactors and high density algal culture techniques, large quantities of groundwater can be stripped of nitrate within a short period of time (Hu et al. 1996; 1998).

The long-term goal of the proposed research was to develop an advanced microalgal system for sustainable large-scale nitrate removal from nutrient-contaminated groundwater. The major objectives of this research grant proposal were to isolate high-performance algal species and to evaluate their nitrate uptake potential under various environmental conditions.

B. Methodology

Isolation and cultivation of microalgae: For isolation of high-performance algal species for maximum nutrient uptake potential, algal samples were collected from various water bodies throughout the metropolitan Phoenix area. Isolation of microalgae, including cyanobacteria, followed the procedure described in Allen (1973). Enrichment cultures for algal isolates were prepared using BG-11 growth medium (Rippka et al. 1979). Membrane filtered (0.45 μm pore size) surface water and groundwater were used for nutrient uptake experiments.

Algal growth measurement: Algal growth was measured using four different methods, depending on the nature of individual species, e.g., unicellular versus filamentous species: optical density, cell count, chlorophyll concentration, and dry weight analysis.

Optical density of the culture was measured with a UV-Vis spectrophotometer at a wavelength of 750 nm.

Cell numbers were determined by placing an aliquot of well-mixed culture suspension on a hemocytometer. Two fields (0.1 mm^3) were counted per each of two hemocytometers. Average of four (4) counts were used to calculate cell concentrations. A linear regression equation between optical density and cell counts was established for individual algal species.

For dry weight measurement, a 20-ml aliquot of culture was filtered through pre-weighed Whatman GF/C filter paper. The filter paper was dried overnight in an oven at 100°C . The difference between the final weight and the weight before filtration was the dry weight of the sample.

A 5-ml culture sample was harvested by centrifugation (14,000 rpm, 5 min), the resulting pellets was extracted with methanol at 4°C overnight. Absorbance of the supernatant at 665 nm was measured with a spectrophotometer.

Nutrient analysis: NO_3^- and PO_4^{3-} measurements were performed on a Bran-Luebbe TrAAcs 800 Autoanalyzer, a continuous flow wet chemistry autoanalyzer using the cadmium reduction method (APHA, #4-89). The instrument was operated according to the standard operating procedure provided by the manufacturer. The standards, QC, and reagents were prepared fresh the day of analysis. For nitrate nitrogen

analysis, the standards were made from a 100 ppm concentration of sodium nitrate ranging from 0.01, 0.02, 0.05, 0.2, 0.8, 2.0, and 5.0 ppm. Every six samples the blank, QC, and drift were measured.

Nitrate uptake rate: Cellular nitrate uptake rate of individual algal species was calculated using the following equation:

$$\text{Nitrate uptake rate (mg N L}^{-1} \text{ h}^{-1}) = (\text{Ln}N_2 - \text{Ln}N_1)/(\text{t}_2 - \text{t}_1);$$

Where t_1 and t_2 represent different time points, and N_1 and N_2 represent nitrate concentration in the growth medium at time t_1 and time t_2 , respectively.

C. Principal Findings and Significance

Isolation of high-performance microalgae

Frequent field sampling trips were made throughout the year to collect algal samples from diverse water environments including groundwater wells, surface canals, urban lakes, irrigation ditches, and wastewater lagoons, as well as private swimming pools. Three unicellular green microalgae, *Chlorella* sp., *Chlorococcum* sp., and *Scenedesmus* sp., one filamentous green alga, *Ulothrix* sp., and one filamentous cyanobacterium, *Pseudanabaena* sp., have been isolated and maintained in the laboratory. The photomicrographs of these algal isolates are shown in **Figure 1**.

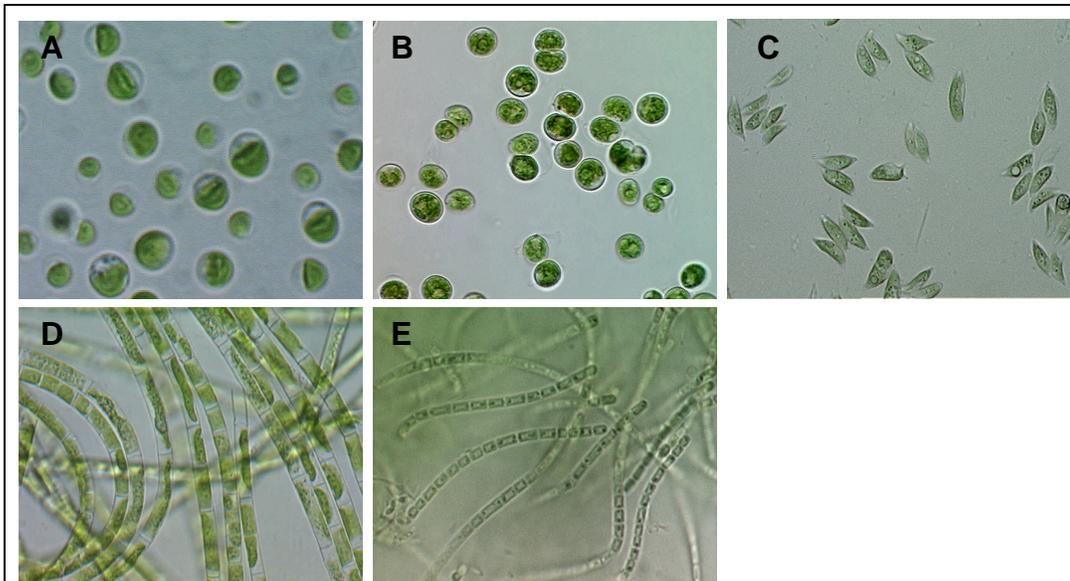
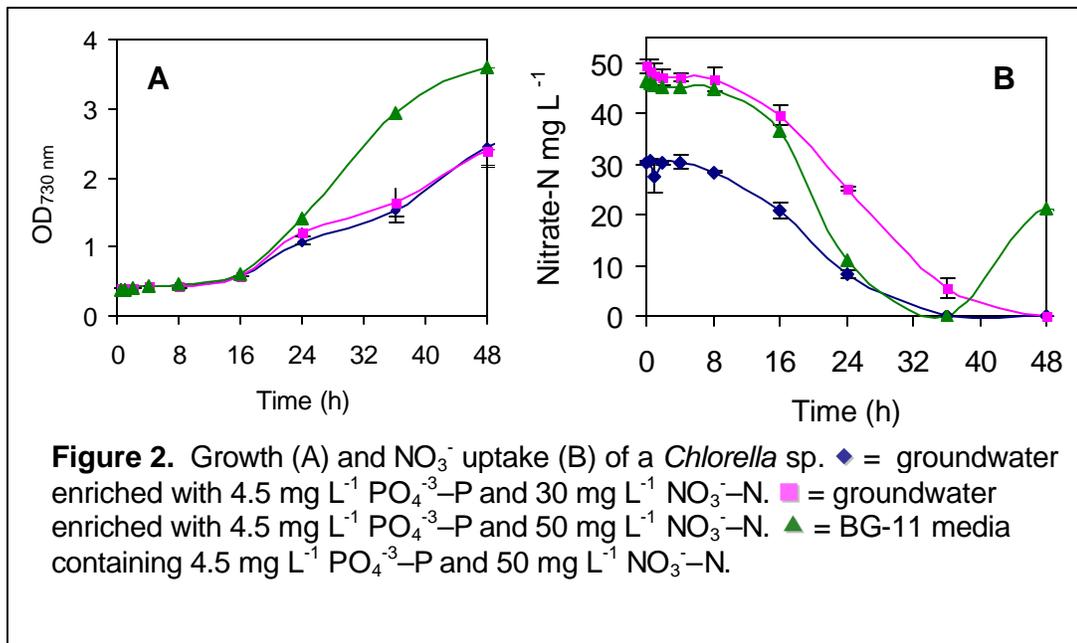


Figure 1. Light photomicrographs of microalgae isolated from metro-Phoenix area. A) *Chlorella* sp., B) *Chlorococcum* sp., C) *Scenedesmus* sp., D) *Ulothrix* sp., and E) *Pseudanabaena* sp.

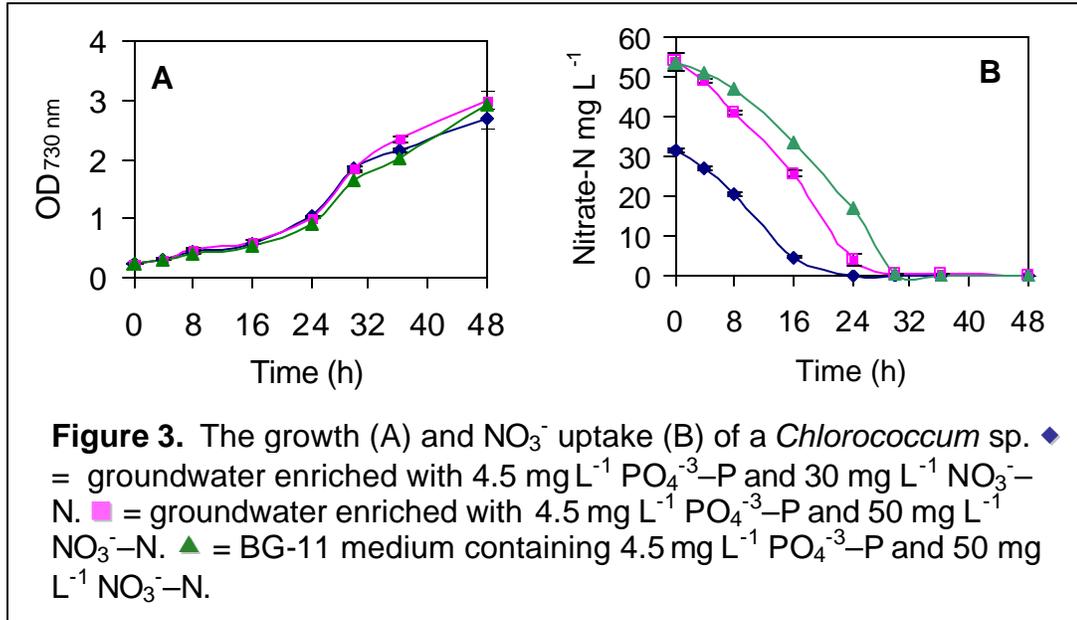
Comparative growth and nitrate uptake kinetics

To characterize high-performance algal species, five algal isolates were compared in terms of growth potential and nitrate uptake rate. All cultures were grown in 300-ml glass column reactors at 25 °C and 185 ? mol m⁻² s⁻¹ light. Aeration was provided by compressed air enriched with 1~2% CO₂ to affect culture mixing. Algae grew in either groundwater or surface water containing 30 to 50 mg L⁻¹ nitrate-N. As the control, BG-11 growth medium was used to support maximum algal growth under the given conditions.

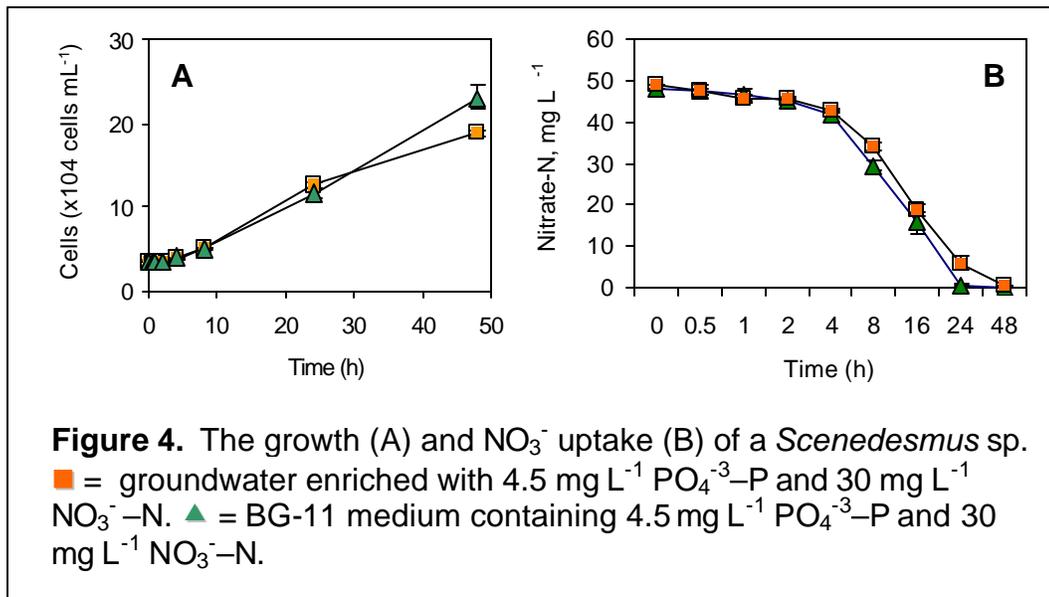
Chlorella sp. – This organism grew well in the groundwater and assimilated nitrate. However, the cells grew more rapidly and removed nitrate faster in the BG-11 artificial growth medium than in the natural groundwater (**Figure 2**). However, 50 mg L⁻¹ nitrate-N was reduced to levels below 10 mg L⁻¹ nitrate-N from the BG-11 growth medium by *Chlorella* cells within first 24 h, whereas 36 h was required to reach the same reduction level using the groundwater as culture medium.



Chlorococcum sp. – Cells exhibited similar growth potential both in groundwater and in the BG-11 growth medium, suggesting higher tolerance of *Chlorococcum* cells to groundwater than observed for *Chlorella* cells. As shown in **Figure 3**, complete removal of 50 mg L⁻¹ NO₃⁻-N occurred in both cultures within 32 h.

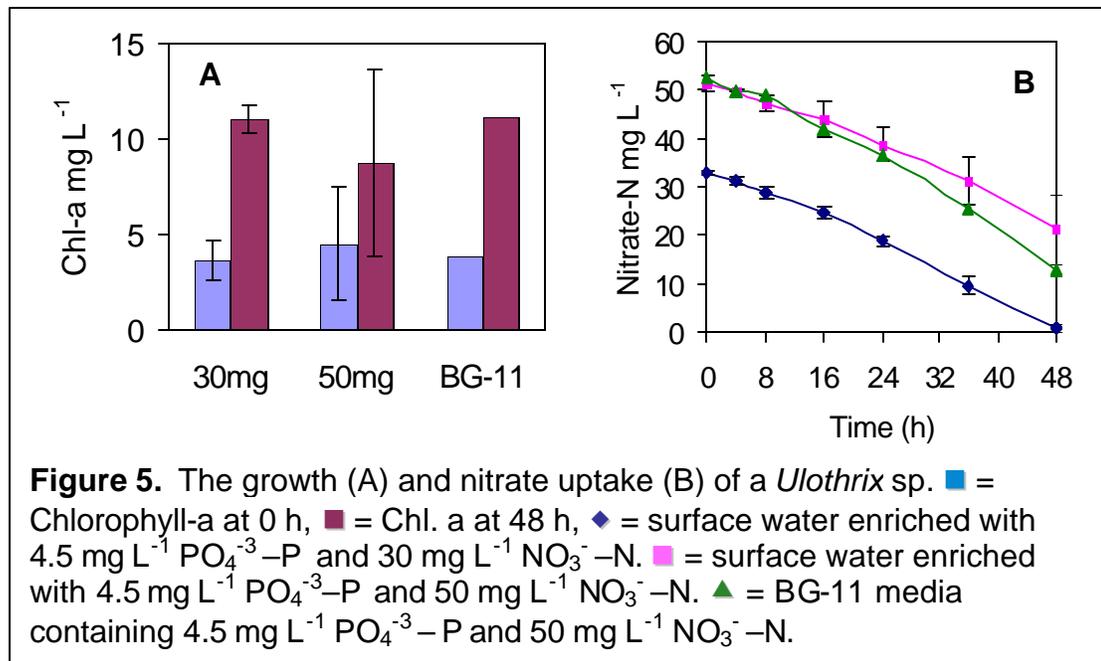


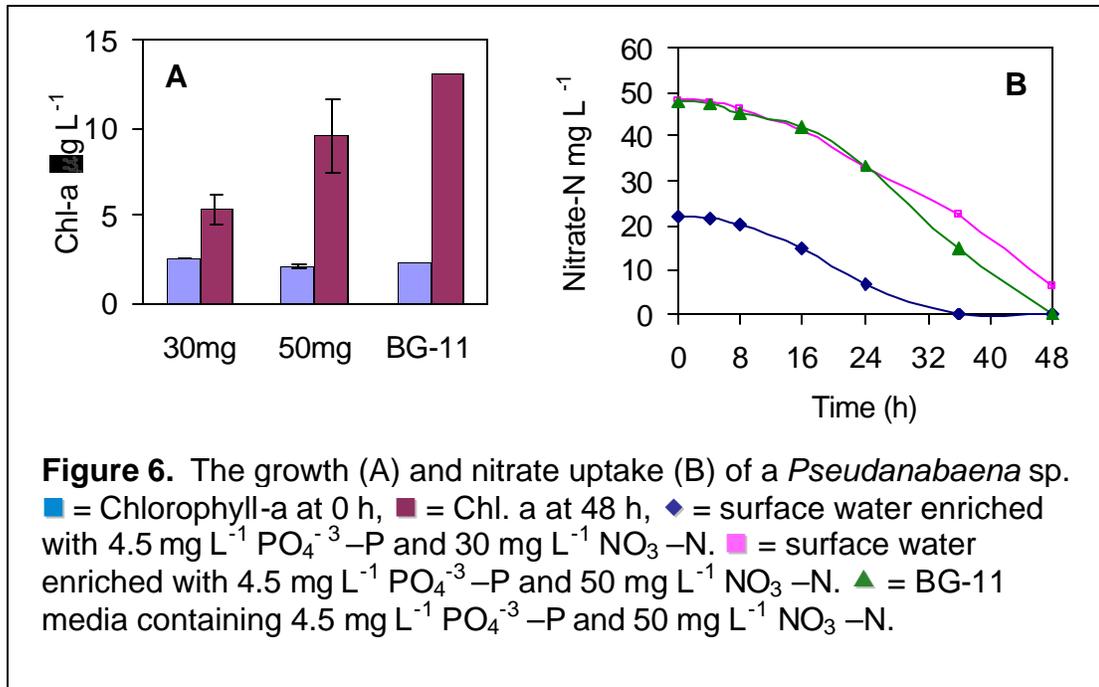
Scenedesmus sp. – Like *Chlorococcum* sp., *Scenedesmus* exhibited similar growth and nitrate uptake rates in the groundwater and the BG-11 growth medium. The nitrate concentration decreased from 50 mg L⁻¹ NO₃⁻-N to below the detection level within 24 h (**Figure 4**).



Ulothrix sp. – This alga exhibited similar growth and nitrate removal potential in surface water and BG-11 growth medium (**Figure 5**). When compared to the unicellular algal species described above, this filamentous alga performed poorly in terms of growth and nitrate removal. For instance, the *Ulothrix* culture resulted in a three-fold increase in biomass over a period of 48 h. In contrast, the *Scenedesmus* culture resulted in nearly nine-fold increase in algal biomass over the same period of time. As a result, by the end of 48 h cultivation period, only about 50% of the 50 mg L⁻¹ NO₃-N was removed in the *Ulothrix* culture.

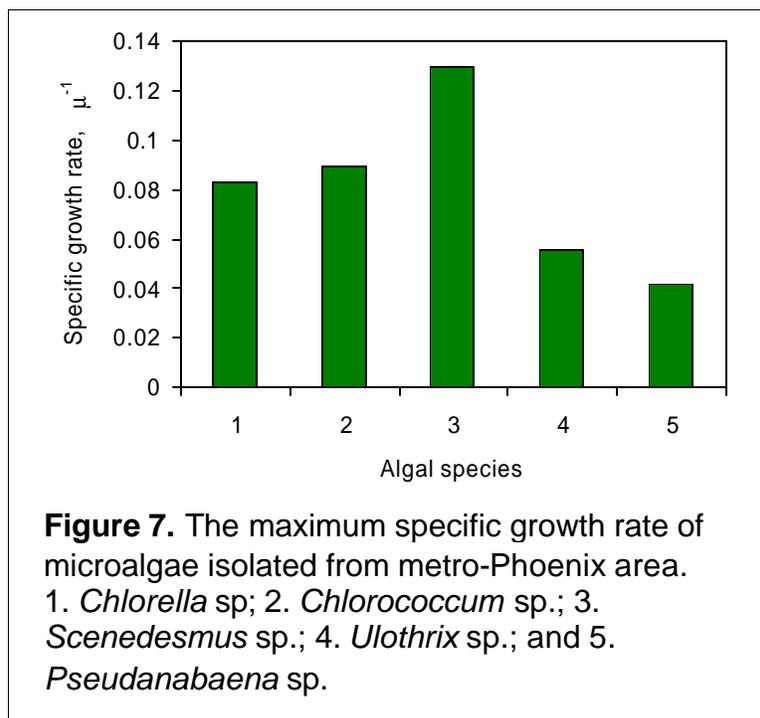
Pseudanabaena sp. – This filamentous cyanobacterial species growth and nitrate uptake rate was similar in the surface water and in BG-11 growth medium (**Figure 6**). The overall performance of the *Pseudanabaena* culture was better than that of *Ulothrix* species in terms of nitrate uptake rate. On the other hand, this species did not perform as well as the unicellular species.





Specific growth rate of isolated algal species

Figure 7 shows the maximum specific growth rates of all five isolated algal species in a batch model under our culture conditions. It demonstrated that the unicellular algal species exhibit higher specific growth rates than the filamentous ones, paralleling the higher nitrate uptake rates. Among the three isolated unicellular green algae, *Scenedesmus* sp. appears to be the more desirable candidate for high performance nitrate removal.

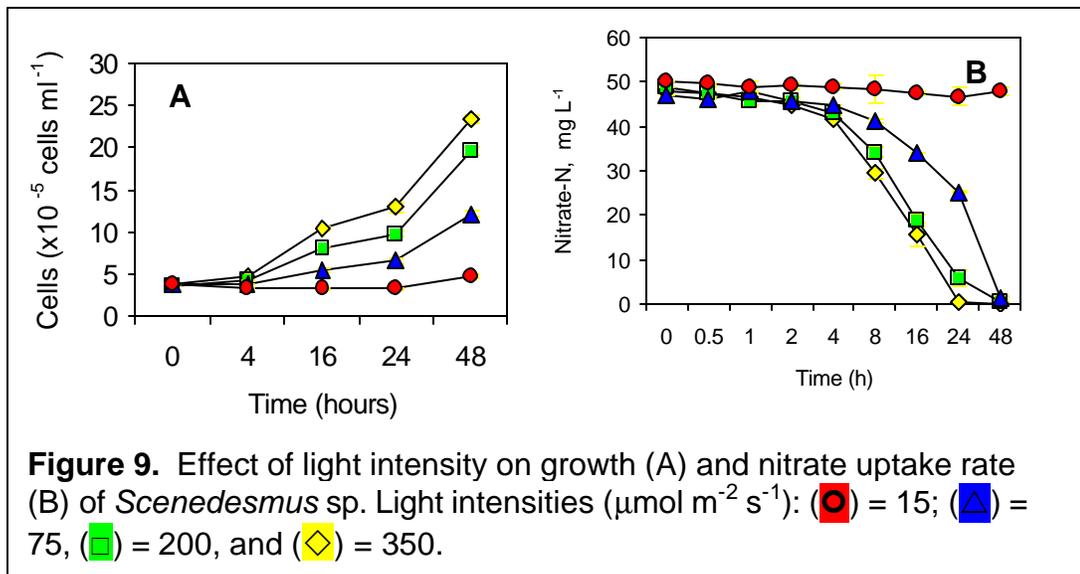
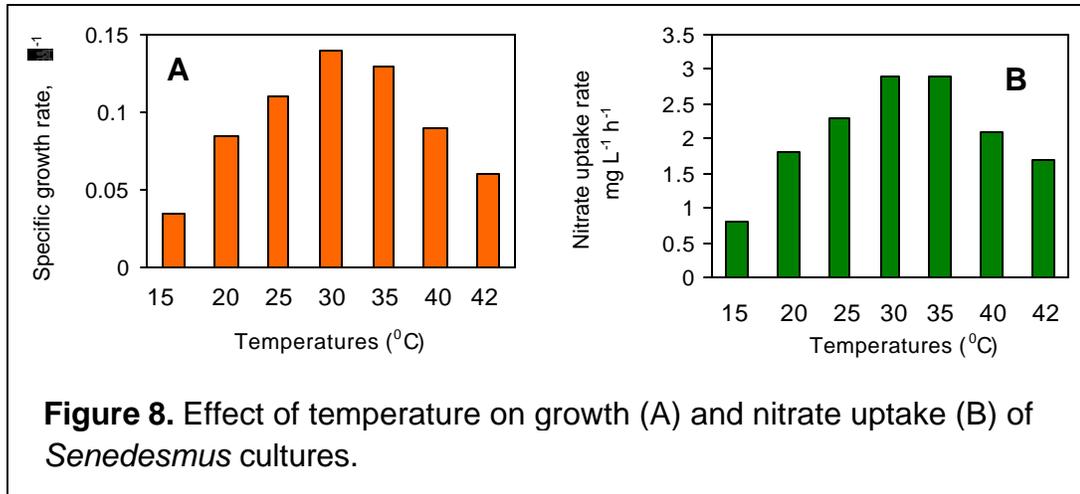


Effect of temperature on growth and nitrate uptake

Scenedesmus sp. was subjected to further investigation to determine the optimal growth temperature and temperature tolerance for nitrate removal. It appears that the alga can tolerate a broad temperature range for growth and nitrate uptake, with the optimal temperature being from 30 to 35 °C which also results in the maximum cellular nitrate uptake efficiency (**Figure 8**). The high temperature tolerance of *Scenedesmus* sp. makes this organism particularly useful for mass culture outdoors in the Phoenix area.

Effect of light intensity on growth and nitrate uptake

As expected there was a positive relationship between light intensity and algal growth and nitrate uptake in cultures of *Scenedesmus* sp. Little growth and nitrate uptake occurred in cultures exposed to 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light. As light intensity increased from 15- to 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$, the maximum specific growth rate increased from 0.035 to 0.12 h^{-1} , resulting in proportional increase in nitrate uptake (**Figure 9**). These results indicate that cellular nitrate uptake is a growth-dependent process: the higher the algal growth rate the higher the cellular nitrate uptake rate. Therefore, any efforts in improving algal growth rates will likely lead to enhancement in nitrate removal.



In summary, four green algae (*Chlorella* sp., *Chlorococcum* sp.; *Scenedesmus* sp., and *Ulothrix* sp.) and one cyanobacterium (*Pseudanabaena* sp.) were isolated from various water environments in metro Phoenix area. Comparative growth and cellular nitrate uptake kinetics were studied among these algal isolates. The specific growth rate ranged from 0.035 to 0.14 h^{-1} with *Scenedesmus* sp. exhibiting the highest growth rate and *Pseudanabaena* sp. the lowest. Compared to the filamentous isolates, the unicellular species exhibited higher specific growth rates. The nitrate uptake rate was species-specific, and hence the algal species that exhibited higher growth rates assimilated nitrate more rapidly. As the high-performance algal strain, *Scenedesmus* sp. was subjected to further investigation, aiming at identifying the optimal culture conditions for sustainable nitrate removal. The specific growth rate and nitrate uptake rate increased with increasing light intensity from 10- to 250 $\mu mol\ m^{-2}\ s^{-1}$. *Scenedesmus* sp. also exhibited a broad temperature tolerance, from 15 to 42 $^{\circ}C$, with 30 to 38 $^{\circ}C$ resulting in the highest nitrate uptake rate. The average nitrate uptake rate

of 2.6 mg N-NO₃⁻ L⁻¹ h⁻¹ which occurred in cultures of *Scenedesmus* sp. is ca. 40% to 150% higher than those reported for nitrate removal by other microalgae and cyanobacteria.

The proposed project objectives have been successfully fulfilled, and the work represents a major milestone in the effort to demonstrate that microalgae have potential as an advanced engineered biological system for large-scale nitrate bioremediation. Continuation of this research is necessary in order to develop a highly efficient and cost-effective large-scale photobioreactor, and to reassess the growth physiology and nitrate uptake potential of *Scenedesmus* sp. under outdoor conditions.

References

Gangolli, S.D., van den Brandt, P.A., Feron, V.J., Janzowsky, C., Koeman, J.H., Speijers, G.J.A., Spiegelhalder, B., Walker, R. and Wishnok, J.S. (1994) Nitrate, nitrite, and N-nitroso compounds. *Eur. J. Pharmacol. – Environ. Tox. And Pharm. Section*, 292:1-38.

Herrero, A. and Flores, E. (1997) Nitrate metabolism, p. 1-33. In A.K. Rai (ed.), *Cyanobacterial Nitrogen Metabolism and Environmental Biotechnology*. Narosa Publishing House, New Delhi.

Hu, Q., Guterman, H. and Richmond, A. (1996) A flat inclined modular photobioreactor (FIMP) for outdoor mass cultivation of photoautotrophs. *Biotechnol. Bioeng.* 51:51-60.

Hu, Q., Kurano, N., Iwasaki, I., Kawachi, M. and Miyachi, S. (1998) Ultrahigh cell density culture of a marine green alga, *Chlorococcum littorale* in a flat plate photobioreactor. *Appl. Microbiol. Biotechnol.* 49: 655-662.

Hu, Q., Westerhoff, P. and Vermaas, W. (2000) Removal of nitrate from drinking water by cyanobacteria: quantitative assessment of factors influencing nitrate uptake. *Appl. Env. Microbiol.* 66: 133-139.

Kapoor, A. and Viraraghavan, T. 1997. Nitrate removal from drinking water - review. *J. Env. Eng.* 123:371-380.

Pontius, F.W. (1993) Nitrate and cancer: is there a link. *J. AWWA*, 85:12-14.

Ward M.H. et al. (1996) Drinking water nitrate and the risk of non-Hodgkin's Lymphoma. *Epidemiol.* 7:465.